

University of Vermont
ScholarWorks @ UVM

UVM College of Arts and Sciences College Honors
Theses

Undergraduate Theses

2016

Effects of dietary restriction and β -hydroxy- β -methylbutyrate supplementation on spontaneous locomotor activity in *Drosophila melanogaster*

Elizabeth Sarah White

Follow this and additional works at: <http://scholarworks.uvm.edu/castheses>

Recommended Citation

White, Elizabeth Sarah, "Effects of dietary restriction and β -hydroxy- β -methylbutyrate supplementation on spontaneous locomotor activity in *Drosophila melanogaster*" (2016). *UVM College of Arts and Sciences College Honors Theses*. Paper 32.

This Undergraduate Thesis is brought to you for free and open access by the Undergraduate Theses at ScholarWorks @ UVM. It has been accepted for inclusion in UVM College of Arts and Sciences College Honors Theses by an authorized administrator of ScholarWorks @ UVM. For more information, please contact donna.omalley@uvm.edu.

Effects of dietary restriction and β -hydroxy- β -methylbutyrate supplementation on spontaneous locomotor activity in *Drosophila melanogaster*

Elizabeth S. White

University of Vermont

Biology Department

Thesis Committee:

Dr. Jim Vigoreaux

Dr. Brent Lockwood

Dr. Karen Lounsbury

Table of Contents

1. Title Page	
2. Table of Contents	
3. Abstract	
4-19. Introduction	
5-7. mTOR Pathway	
7-9. Sirtuins	
9-10. Dietary Restriction (DR)	
10-11. β -hydroxy- β -methylbutyrate (HMB)	
11-12. Aging and Lifespan	
12-13. Spontaneous Activity in Laboratory <i>Drosophila</i>	
14. <i>Drosophila melanogaster</i> as a Model Organism	
14-15. This Study	
16-19. Methods	
16. Fly Stocks and Treatments	
17-18. Spontaneous Flight and Walking Measurements	
19. Data Analysis	
20-28. Results	
20-22. Single Diet Measurements	
23- 25. Switching Diet Experiments: One Week	
26-28. Switching Diet Experiments: Five Weeks	
29-38. Discussion	
31-34. Dietary Restriction	
34-35. HMB	
35-36. Possible Sources of Errors	
36-38. Future Studies	
39-43. References	
44-45. Appendix I: Seahorse Protocol	
46-48. Appendix II: Development of Seahorse Protocol	

Abstract

Dietary restriction (DR) has been shown in a wide variety of model organisms, including *Drosophila melanogaster*, to increase lifespan and health span. It is unclear how much of this effect is due to a DR-induced increase in physical activity. β -hydroxy- β -methylbutyrate (HMB) supplementation has been shown to improve health span in *Drosophila*. The exact mechanism of this effect has not yet been elucidated but it is possible that, like DR, HMB increases health span through increased physical activity.

In this study, the spontaneous flight activity (SFA) and spontaneous walking (activity) was measured at one and five weeks after eclosion for flies raised on low yeast, HMB-supplemented, and normal laboratory diet. To see if any effects of diet were immediate or occurred from life-long consumption, flies were switched to a new diet and their SWA and SFA was measured. Also, to see if age had any effects on the association between diet and physical activity, SFA and SWA were measured in flies one week (young) and five weeks (old) after eclosion. We hypothesized that (i) HMB and dietary restriction will increase SFA and SWA activity compared to a standard laboratory diet in young flies and that the effect of dietary restriction would be immediate; and (ii) HMB and dietary restriction will increase SFA and SWA compared to a standard laboratory diet in old flies and that this effect would be more pronounced than that in younger flies.

No association between spontaneous activity and diet was found, either in young flies or in older flies. In switching experiments, no significant change in spontaneous activity was found, concluding that there was no immediate or long-term effect of diet on physical activity. From this, we concluded that DR-induced increased lifespan and HMB-induced increased health span are likely not due to increased physical activity.

Introduction

Aging causes a shift in equilibrium between protein breakdown and synthesis to favor protein breakdown, or proteolysis. This is due to a wide variety of causes but is largely attributed to two main pathways: mechanistic target of rapamycin (mTOR) pathway and sirtuins. In both of these cases, dietary restriction has been shown to act through these pathways to extend lifespan in a wide variety of eukaryotic organisms [1,2]. Dietary restriction has also been shown to increase physical activity [3]. A major question of dietary restriction is how its effects relate to increased physical activity.

More recently of interest as a possible life and health span extension dietary intervention has been supplementation of the leucine metabolite, β -hydroxy- β -methylbutyrate (HMB). While the exact mechanism of action of HMB has not yet been elucidated, it appears to upregulate mTOR [4]. While HMB and dietary restriction have opposite effects on mTOR, they have similar effects on *Drosophila melanogaster*, extending health span through the attenuation of flight loss and possibly also extending lifespan [5,6]. This study will further investigate the role that physical activity, or, more specifically, spontaneous flight activity, plays in both of these dietary interventions. Also, since mTOR is downregulated with old age, this study will compare any changes in physical activity of young (one week old) versus old (5 week old) flies on a restricted, standard, and HMB-supplemented diet.

Target of Rapamycin (TOR) Pathway

Proteostasis, or protein homeostasis, can be defined as a balance between the degradation and synthesis of proteins in a biological system. Proteolysis is critical for the removal of damaged or improperly folded proteins. These faulty proteins are typically flagged for degradation by the attachment of a small protein called ubiquitin.

Ubiquitination is initiated by an enzymatic cascade involving ubiquitin-activating enzymes (E1s), ubiquitin conjugating enzymes (E2s), and then ubiquitin protein ligases (E3s) [7]. Once ubiquitinated, proteins are targeted by the ubiquitin-proteasome system (UPS) or the autophagosome-lysosome pathway. In the ubiquitin-proteasome pathway, ubiquitinated proteins are hydrolyzed by the proteasome, an enzyme complex in eukaryotic nuclei and cytoplasm [8]. In the autophagosome-lysosome pathway, autophagosomes deliver damaged proteins and organelles to lysosomes where they are degraded. Autophagy functions as a stress response that is upregulated by starvation or oxidative stress. The magnitude of autophagosome formation is regulated by amino acid sufficiency via the target of rapamycin (TOR) pathway signaling [9].

TOR is a nutrient-sensing kinase that phosphorylates several proteins, including Protein kinase B. Protein kinase B, or Akt, then phosphorylates the transcription factor FoxO, thus inactivating it. When activated, FoxO upregulates the expression of ubiquitin ligases like MURF-1 and atrogin-1, thus increasing ubiquitination. This increase in ubiquitination accelerates protein degradation. Therefore, activation of TOR ultimately causes a decrease in protein degradation. TOR also phosphorylates S6K1 and 4E-BP1, causing an increase in protein synthesis [10]. In this manner, TOR signaling increases cell growth and metabolism, therefore indirectly decreasing the amount of energy

devoted to the maintenance of somatic cells (Figure 1). Decreased somatic cell maintenance leads to a buildup of damaged proteins, tissue deterioration, decreased tissue functionality, and, ultimately, a shortened lifespan [11].

Regulation of TOR has been implicated in lifespan determination. In the fruit fly *Drosophila melanogaster*, dominant negative (inactive) alleles of TOR or S6 kinase and a hypomorphic (reduced activity) allele of TOR has also been shown to increase lifespan in the fruit flies [12]. In the budding yeast *Saccharomyces cerevisiae*, lifespan is measured by replicative (number of proliferations during a mother cell's lifetime) and chronological (amount of time that a cell can survive in a quiescent state) lifespans. Both replicative and chronological lifespans of *Saccharomyces cerevisiae* were significantly extended with a deletion of TOR1 [13,14].

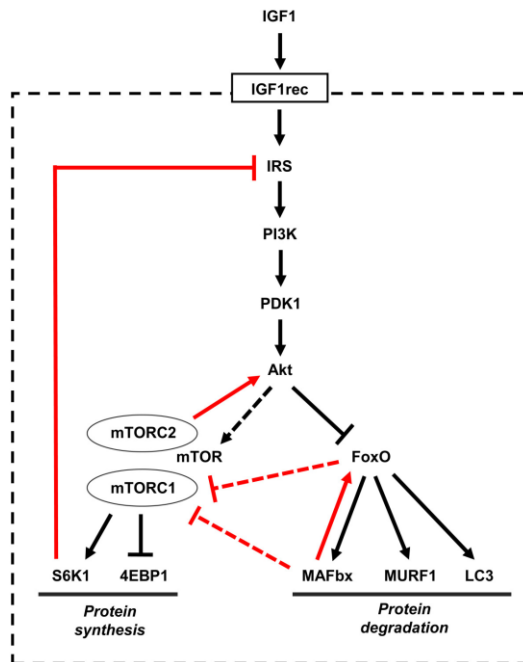


Figure 1: A simplified overview of the mTOR pathway, adapted from Schiaffino, 2011 [15]. Here, pointed arrows indicate activation while “T-shaped” arrows indicate inhibition. Dotted arrows indicate that the effect is hypothesized, with some data supporting the hypothesis, but the effect has not been proven. Solid arrows indicate the effect is known and well documented.

Sirtuins

Sirtuins (Sirt) are signaling proteins that primarily function to improve somatic cell maintenance (Figure 2) [16]. Unlike mTOR, sirtuin activity is increased in times of dietary restriction [17]. In this manner, mTOR and sirtuins act as opposing forces in the effects of nutrient intake on somatic cell maintenance. Where mTOR decreases maintenance in times of amino acid sufficiency, sirtuins increase maintenance in times of low amino acid intake. There is even some crosstalk between the two pathways, as the acetylation of S6K1 blocks mTORC1 phosphorylation and acetylation of S6K1 is inhibited by both SIRT1 and SIRT2 [18].

Sirtuins were first discovered in *Saccharomyces cerevisiae* as “silencing factors.” Termed Sir for “silence mating-type information regulator,” these enzymes deacetylate histones to decrease access to genes involved in autophagy, protection from oxidative damage, and improved DNA repair [19]. All of these effects are involved in somatic cell maintenance, which can improve the lifespan of an organism. Overexpression of Sir2 has been shown to increase lifespan in *Saccharomyces cerevisiae*, *Caenorhabditis elegans* and *Drosophila melanogaster* [19, 20, 21].

In mice, Sirt-1 has been shown to induce autophagy through the deacetylation of essential autophagic factors such as ATG5 and ATG7 [22]. Increased autophagy (through pharmacological or genetic means) has been shown in many other studies to improve longevity [23, 24]. In *Drosophila melanogaster*, overexpression of dSir2 or activation of dSir2 by the drug resveratrol increased lifespan. Interestingly, one study conducted by Parashar et al found that the extension of lifespan they found with dietary restriction disappeared in dSir2 null flies. The group also found that flies fed the restricted diet (decreased protein) displayed an increased amount of spontaneous physical activity. This increase in physical activity was lost in the dSir2 null flies, indicating that sirtuins are responsible for the increased physical activity observed in organisms fed a restricted diet [25].

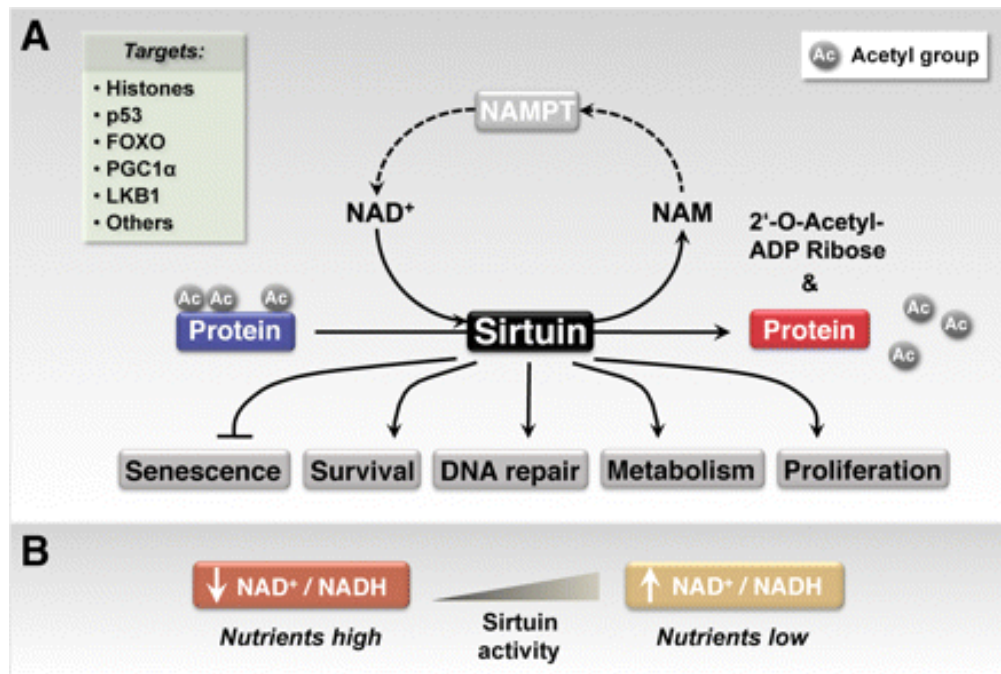


Figure 2: A simplified overview of sirtuin pathways, adapted from Oellerich 2012 [17]. Part A depicts a NAD⁺-dependent deacetylation of various protein targets. Some of these targets include p53, which regulates cell cycle in response to various stressors; histones, which regulate gene access; and FOXO, which triggers apoptosis but is inactivated by sirtuins. Part B depicts the nutrient dependence of sirtuins. When nutrients are high, sirtuin activity is low. When nutrients are low, sirtuin activity is high.

Dietary Restriction

Dietary restriction (DR) has been shown in a wide variety of eukaryotic organisms to extend lifespan [26]. DR can be defined as simply a decrease in total caloric intake or, more specifically, a decrease in total protein intake. Decreased protein intake has some effects through the mTOR pathway as mTOR senses amino acid sufficiency. Decreased mTOR signaling causes decreased protein synthesis and, as is the case with aging, can cause sarcopenia and muscle atrophy. However, downregulation of mTOR also allows more energy to be allotted to somatic cell maintenance. Increased somatic cell

maintenance can extend life and health spans by avoiding the cytotoxic accumulation of poly-ubiquitinated protein aggregates [27].

In the nematode *Caenorhabditis elegans*, TOR RNAi (interrupting RNA, which decreases expression of the TOR gene) extended the mean lifespan by about 27%. However, when comparing *C. elegans* just fed a restricted diet to those fed a restricted diet and treated with TOR RNAi, there was no significant difference in mean lifespan. This finding indicated that dietary restriction likely acts through the TOR signaling pathway [1].

Dietary restriction also acts through sirtuins. This mechanism of action was first discovered in yeast, when researchers observed a loss of lifespan extension from dietary restriction in Sir2 loss-of-function mutants [28]. A similar effect has been observed in *Drosophila melanogaster*, rats, and rhesus monkeys [5, 29, 30].

β -hydroxy- β -methylbutyrate (HMB) supplementation

β -hydroxy- β -methylbutyrate (HMB) is a metabolite of leucine that, when added as a supplement to the diets of malnourished elderly adults was found to decrease mortality and improve nutritional status after a 90-day trial period [31]. In humans, HMB is currently being studied as a possible nutritional aid for elderly patients and AIDS and cancer patients with wasting syndrome.

In 2013 a human study on HMB supplementation to improve recovery in male athletes after resistance training was conducted. Indices of muscle damage, protein breakdown, recovery, and hormone status were measured before and after resistance

training. The group found a significant improvement in perceived recovery status (PRS) and decreased muscle protein breakdown after resistance training with HMB supplementation. These findings indicate that HMB may possibly be used as an ergogenic aid for athletes [32].

In *Drosophila melanogaster*, HMB supplementation has been discovered to have similar effects as dietary restriction in attenuation of flight loss, though the exact mechanism has not yet been elucidated [5]. These findings seem a bit contradictory as HMB has been thought to upregulate TOR by increasing the phosphorylation of Akt. Akt, which deactivates TOR when active, is inactivated by phosphorylation [33]. One hypothesis to explain this seemingly contradictory finding is that consumption of HMB, like dietary restriction, increases physical activity.

Aging and Lifespan

Aging is defined as a decline or deterioration in bodily functions over time. While many studies focus on the lifespan of an organism, fewer look at the health span, or how long the organism maintains normal, healthy functioning. This is important as aging changes the balance of proteostasis by causing an increase in proteolysis and a decrease in protein synthesis. This causes a net loss of proteins, often leading to sarcopenia and atrophy in muscle tissues. Sarcopenia refers to an aging-dependent degenerative loss of muscle tissue [34]. Muscle atrophy refers to a decrease in total muscle mass, typically due to inactivity, malnutrition, and/or aging. Muscle atrophy is a part of sarcopenia. By

observing health span in relation to muscle functioning in *Drosophila*, we can observe the effects of a wide variety of interventions on sarcopenia and muscle atrophy with age.

Senescence, or the process of deterioration with age, is unavoidable but its progression can be altered via both mTOR and sirtuin pathways [35]. As mentioned before, decreased mTOR and increased sirtuin signaling leads to increased lifespan, likely indicating a slowed rate of deterioration. This slowed rate of deterioration is also indicative of an increased healthspan.

Spontaneous Activity in Laboratory *Drosophila*

It has been shown that many of the effects of dietary restriction (DR) are likely due to an increase in physical activity [36, 37]. To investigate this topic, a study conducted by Partridge et al on *Drosophila* measured the spontaneous locomotor activity (SLA) of flies using a light beam. Spontaneous locomotor activity refers to any amount of movement (flying or walking) done by a fly not due to external stimuli. The group counted the number of times the light beam was broken to estimate the amount of locomotor activity done by a cohort of flies. The SLAs of flies on a restricted versus standard diet were compared. This experiment, like other on organisms fed a restricted diet, found an increase in SLA with DR [6]. The main downfall of measuring SLA through this method is that it is unable to distinguish between walking and flying flies. This is a critical distinction to make as flying is far more metabolically costly and causes more oxidative damage than walking. In mammalian models, moderate physical activity has been shown to increase lifespan and improve muscle function [38]. Less is known

about the effects of more intense physical activity but many studies suggest that intense physical activity shortens lifespan.

Another study, conducted by Magwere et al altered the amount of total living space a fly had access to without altering the volume per fly [39]. This study found that the flies with less living space took off (began flying) less often and lived longer. The extension of lifespan was largely attributed to a decreased amount of lipoxidative damage, supposedly due to a decreased amount of flight. While this study was able to decrease how often flies began flying, it was unable to measure how long flies spent flying or how much walking they were doing. The study also did not account of the decreased amount of surface area per fly in the larger flask. While the group made sure both treatments had the same volume per fly, they could not adjust for effects of different bottle surface area per fly.

Another recent study on *Drosophila* has shown a loss of DR-induced increased lifespan with ablation of wings, suggesting that physical activity, in particular, flight, is crucial for DR-induced lifespan extension [3]. What this study failed to account for are the possible effects that the loss of the wings may have on the lifespan of the organism. It is very likely that the decrease in lifespan between flies on a restricted diet without wings versus those on a restricted diet with wings was primarily due to the actual loss of wings and not the decrease in flight activity.

Drosophila melanogaster as a Model Organism

Drosophila melanogaster have been used as a model organism for research of genetics and genetic control of development and behavior for the past century [36]. Due to this, the genome of *Drosophila* is fully sequenced and well known. *Drosophila* also have a short lifespan (usually 6-10 weeks) so experimental changes in lifespan can easily be observed. *Drosophila* can be easily maintained in large numbers, allowing for larger sample sizes than would be possible for other model organisms, like mice or rats.

More recently, *Drosophila* has been used as a model for metabolic diseases and age-related loss of muscle function [41, 42]. Since many pathways involved in muscle growth and maintenance (like TOR and sirtuins) are relatively conserved from *Drosophila* to humans, any findings in studies on *Drosophila* muscle could lead to studies on humans [43, 44].

This Study

The aim of this study was to analyze the effects of HMB supplementation and caloric restriction (versus a standard laboratory diet) on spontaneous locomotor activity in one and five week old flies, distinguishing between walking and flying activity. We hypothesized that (i) HMB and dietary restriction will increase spontaneous flight (SFA) and walking (SWA) activity compared to a standard laboratory diet in young flies and that the effect of dietary restriction would be immediate; and (ii) HMB and dietary restriction will increase SFA and SWA compared to a standard laboratory diet in old flies and that this effect would be more pronounced than that in younger flies.

For this study, SFA and SWA were measured in a large cohort of flies to estimate how much physical activity was done by flies fed certain diets. Up to this point, no studies have been able to distinguish between the amount of walking (moderate exercise) and flying (vigorous exercise) a fly is doing over the course of the day. This experiment will be one of the first to measure specifically the amount of flight and walking being done by a population of flies each day. Average total flight done per fly per day as well as average length of each individual flight will be measured.

Methods

Fly Stocks and Treatments

These experiments were conducted on lab-reared wild type *Drosophila melanogaster*. Flies were age-matched by first allowing 100 healthy, one- to three-week old flies to lay eggs in 500 mL plastic flasks with 50 mL of food for three days. When flies eclosed, the flask was completely emptied of flies using carbon dioxide, and the remaining pupa were allowed to eclose for two days. Flies were collected again using carbon dioxide. All flies collected eclosed within a 48 hour period of each other. Fly bottles were stored in an environmental room at 23 degrees Celsius and 70% humidity. Flies were transferred to new bottles every three days.

Three treatments were created, each beginning with 600 flies per treatment (Figure 3). The first treatment was the control, where flies are fed a standard lab diet. The second treatment was the HMB supplemented diet, where flies are fed a normal diet but with 10mg/mL HMB. The third treatment was a calorie-restricted diet where flies were fed food with a 75% reduction in yeast (protein) concentration.

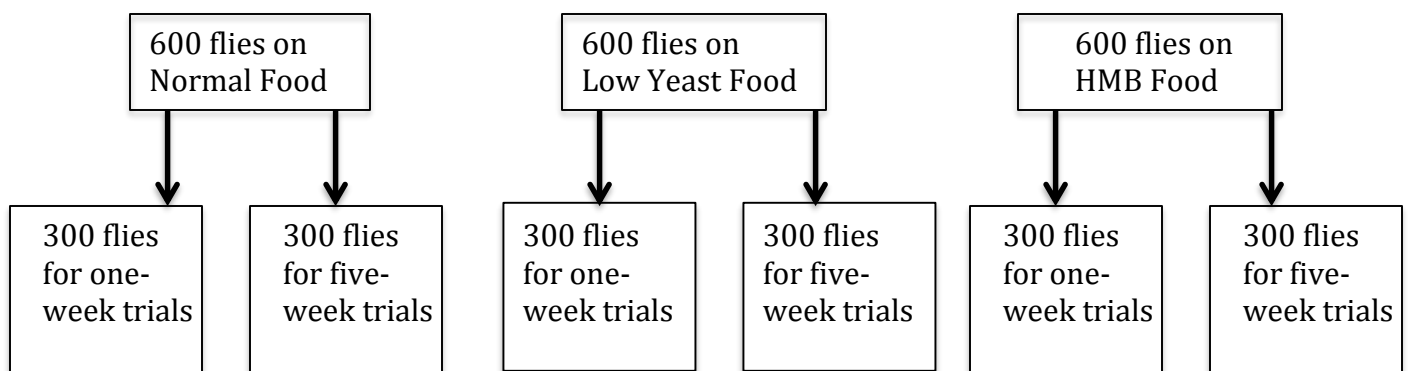


Figure 3: Flow chart depicting the designation of flies for experimental groups. All three groups started with 600 flies (total of 1800 flies). 300 were designated to one-week trials (100 flies per trials) and 300 were designated to five-week trials (100 flies per trials).

Spontaneous Flight and Walking Measurements

Spontaneous flight and walking activity were measured using a clear plexiglass cylindrical chamber surrounded by infrared LED infrared lights. The chamber was kept in the lab, surrounded by light-blocking black and white curtains. Humidity was maintained at 70% inside the chamber with a plastic tube connected to a bubbler. Three circles of food were at the base of the chamber, each about one inch in diameter. Above the cylinder was a camera with an infrared light filter (Figure 4).

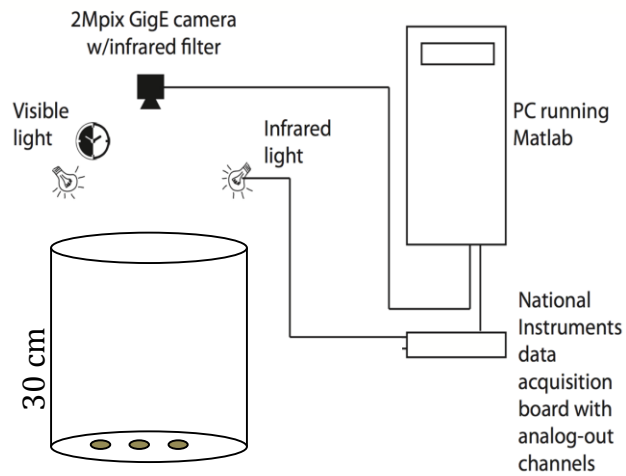


Figure 4: Schematic of the apparatus used for measuring walking and flying activity in a population of flies. The chamber (bottom left) contains three circular wells of food at the bottom and is surrounded by infrared lights which are controlled by a National Instruments data acquisition board with analog-out channels. Above the chamber is a camera with an infrared filter, also connected to the National Instruments data acquisition board with analog-out channels and a PC running MatLab.

One hundred flies were put into the chamber and allowed to equilibrate for 24 hours. Photographs were then taken at a rate of 11 frames per second for 9 seconds every 5 minutes over the course of 24 hours (12 light and 12 dark). After 24 hours on the flies' native diet (the diet on which they had eclosed and been raised on), the diet was switched to another type of diet and measurements were made for the next 24 hours (Figure 5). Normal food flies were switched to low yeast food and low yeast and HMB food flies were switched to normal food.

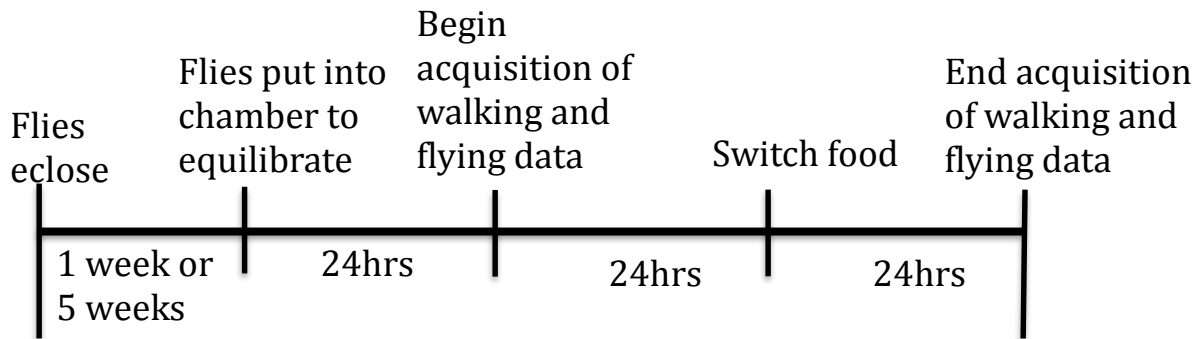


Figure 5: Timeline of each trial. Flies were aged to either one or five weeks after eclosion on their designated diet. Flies were put into the cylindrical chamber for 24 hours to equilibrate. Data was collected for the next 24 hours. The food was switched after 24 hours and another 24 hour measurement was made.

Data Analysis

In the photographs collected, black pixels were assigned a zero and white pixels were assigned a value of 255. Grey pixels were assigned a number somewhere between 255 and zero, depending on their brightness. The change in pixel brightness between two frames was found by subtracting the second frame from the one before it. By doing this, the computer could tell if a fly was walking, flying, or staying still. Using MatLab we were able to determine the number of flies flying, walking, and not moving at any given time point throughout the day. From these data, the amount of total flight and walking time over the 24-hour period was calculated and divided by the total number of flies in the chamber to find seconds of spontaneous flight or walking activity per fly per 24 hours.

A paired t-test was conducted to compare the average flight and walking per fly for each of the switching experiments using Statplus. A one-way analysis of variance (ANOVA) with a Tukey's multiple comparison test was used to compare the average flight and walking per fly for each of the treatments' first 24-hour measurements using Graphpad Prism. P-values less than or equal to 0.05 were considered statistically significant.

Results

Single Diet Measurements

At one week old, there was also no statistically significant difference between low yeast and normal ($p = 0.892$) or normal and HMB ($p = 0.999$) flies' average flight time for the first 24 hours (Figure 6). There was also no statistical difference between low yeast and normal ($p = 0.397$) or normal and HMB ($p = 0.975$) flies' average walking time for the first 24 hours (Figure 7).

For five-week old flies, there was also no statistically significant difference between low yeast and normal ($p = 0.144$) or normal and HMB ($p = 0.092$) average flight time for the first 24 hours (Figure 8). There was no statistical difference between low yeast and normal ($p = 0.497$) or normal and HMB ($p = 0.278$) flies' average walking time for the first 24 hours (Figure 9).

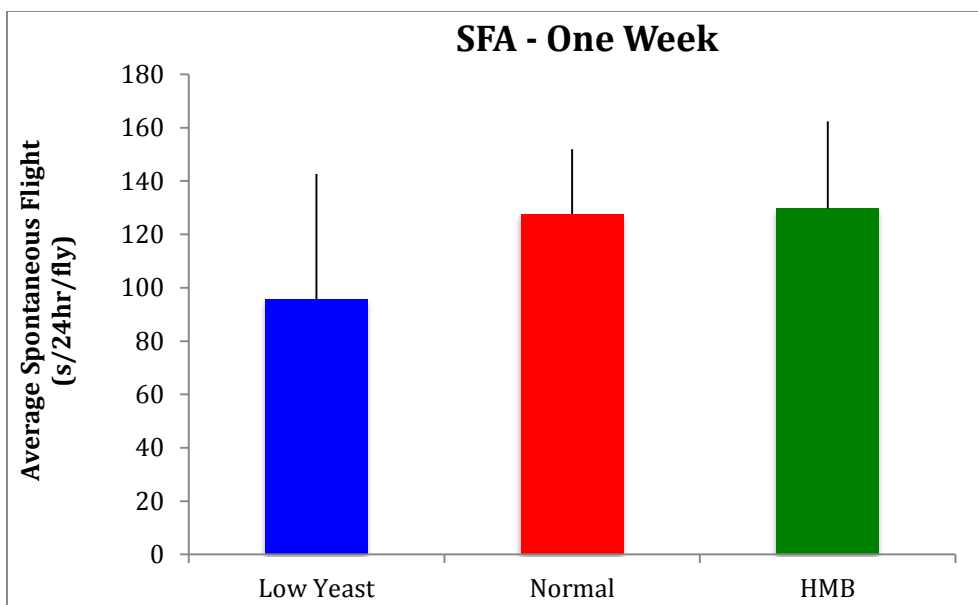


Figure 6: Bar graph with standard error bars of spontaneous flight activity (SFA) for each dietary intervention at one week old (N = 3). Y-axis represents average flight time in seconds per fly per 24 hours.

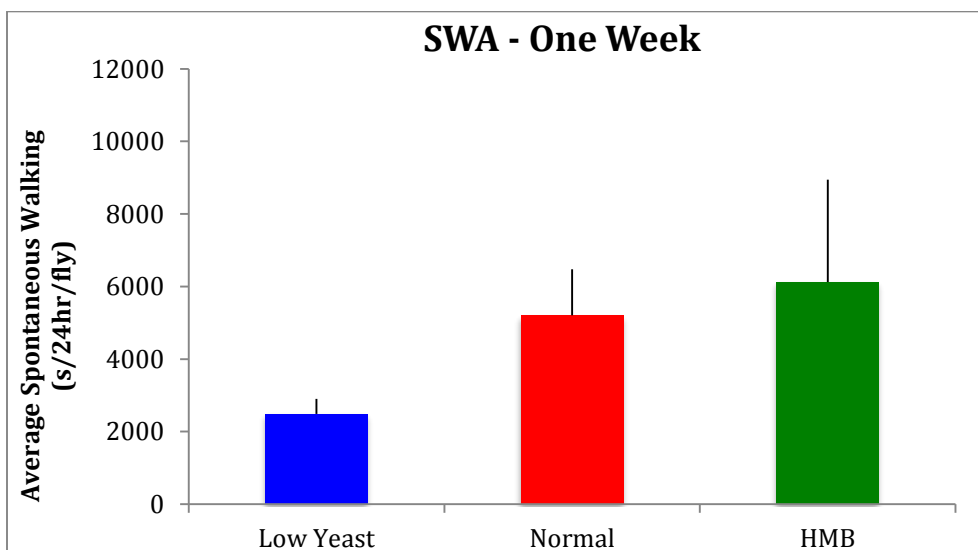


Figure 7: Bar graph with standard error bars of three spontaneous walking activity (SWA) for each dietary intervention at one week old (N = 3). Y-axis represents average flight time in seconds per fly per 24 hours.

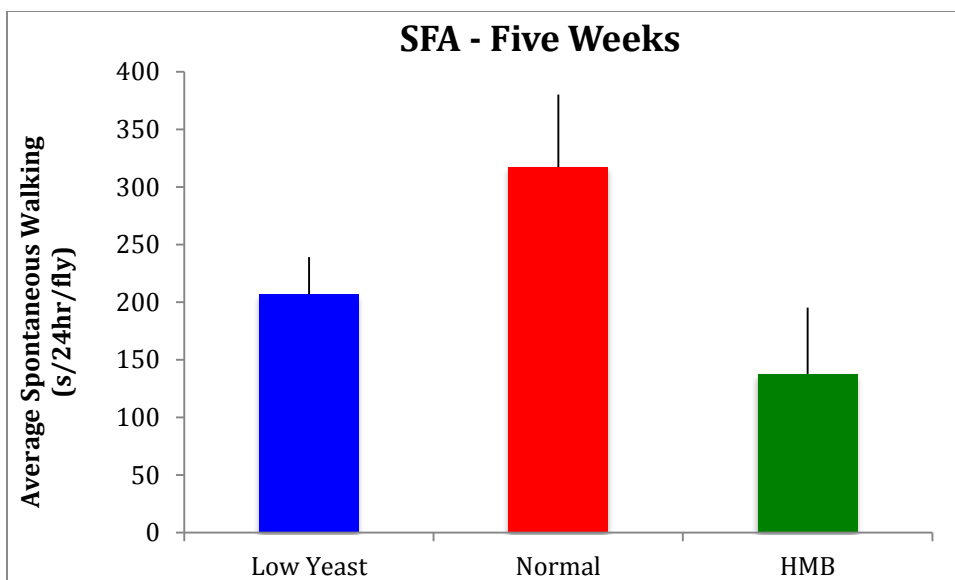


Figure 8: Bar graph with standard error bars of spontaneous flight activity (SFA) for each dietary intervention at five weeks old (N=3). Y-axis represents average flight time in seconds per fly per 24 hours.

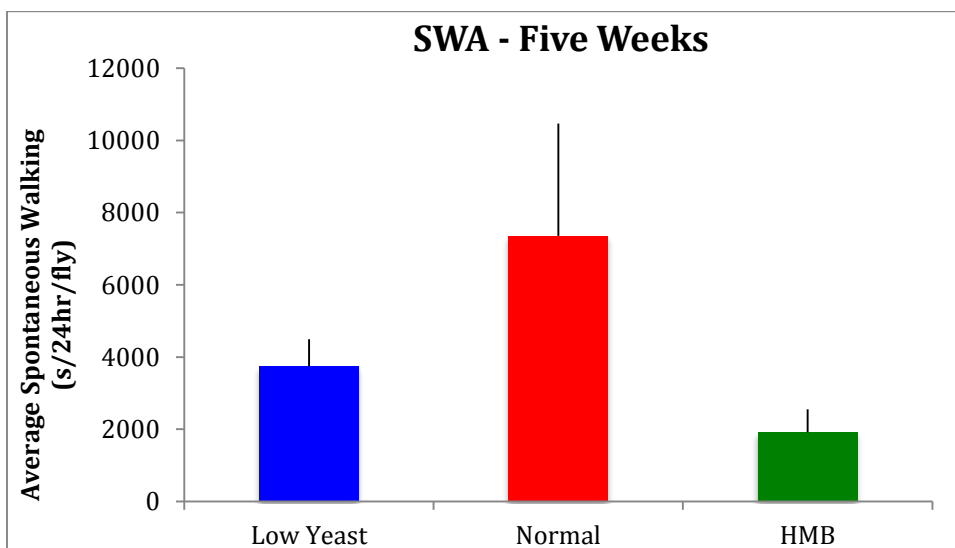


Figure 9: Bar graph with standard error bars of three spontaneous walking activity (SWA) for each dietary intervention at five weeks old (N=3). Y-axis represents average flight time in seconds per fly per 24 hours.

Switching Diet Experiments: One Week

In a comparison of spontaneous activity by low yeast flies on low yeast food and then switched to normal food, there was no significant change in flight activity (95.50 ± 47.2 vs. 58.16 ± 6.74 ; $p = 0.482$) or walking activity (2493 ± 410.0 vs. 2068 ± 152.7 ; $p = 0.103$) (Figure 10A and 11A, Table I and II). Normal-fed flies that were switched to low yeast food showed no change in flight activity (127.6 ± 24.3 vs. 132.2 ± 34.7 ; $p = 0.939$) or walking activity (5202 ± 1272 vs. 2152 ± 496 ; $p = 0.194$) (Figure 10B and 11B, Table I and II). When switching from HMB to normal food, there was no significant difference in spontaneous flight activity (129.7 ± 32.7 vs. 133.9 ± 25.6 ; $p = 0.635$) or walking activity ($p = 0.990$) (Figure 10C and 11C, Table I and II).

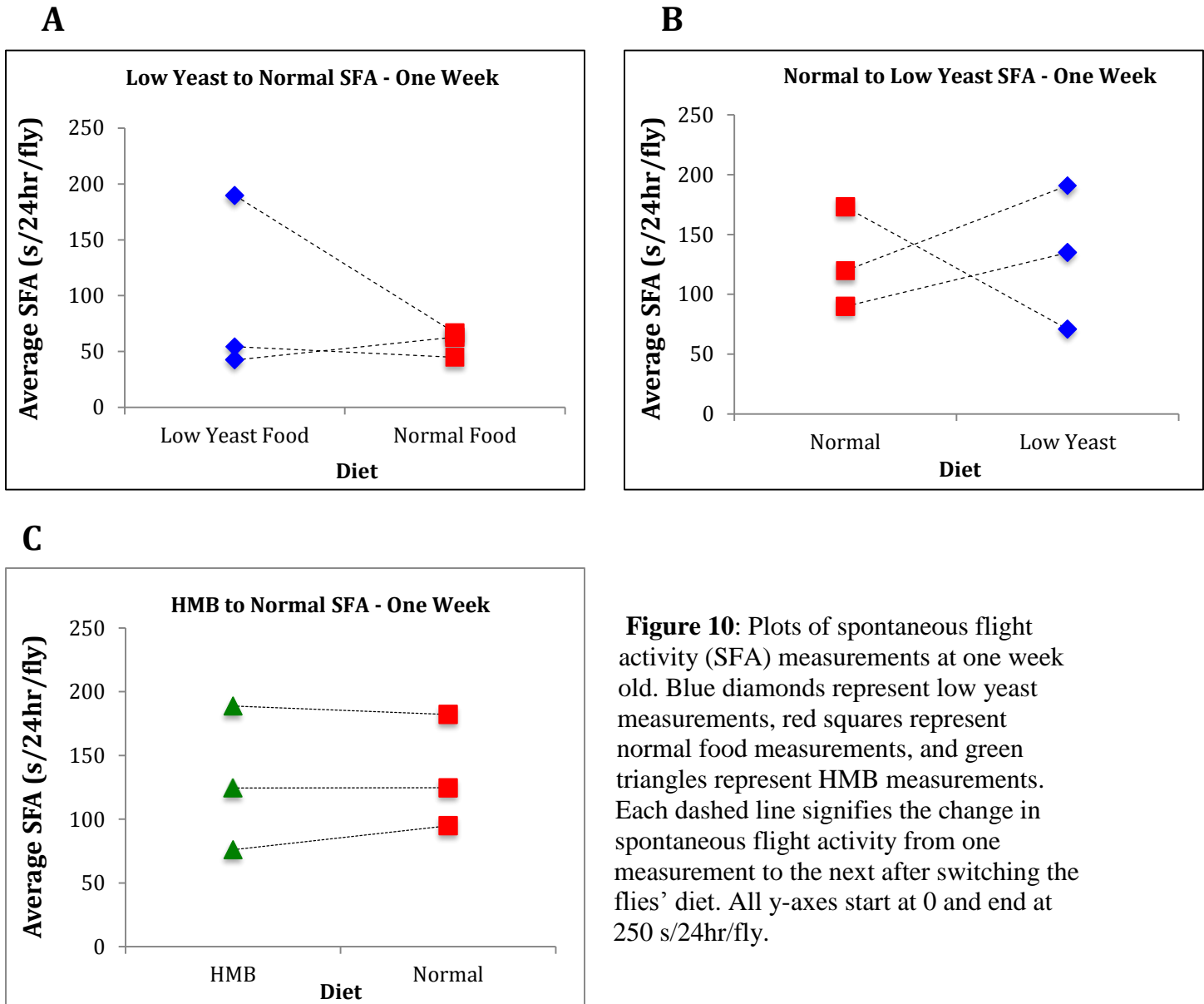


Table I: Average spontaneous flight activity (SFA) times (in seconds per fly per 24 hours) for switching experiments at one week old. P-values from a paired t-test are given for each experiment.

SFA - 1Wk	Switched to	
Native Diet	LY	Nor
LY	NA	Average LY = 95.50, Average Nor = 58.16; p = 0.482
Nor	Average Nor = 127.6, Average LY = 132.2; p = 0.939	NA
HMB	NA	Average HMB = 132.4, Average Nor = 138.6; p = 0.712

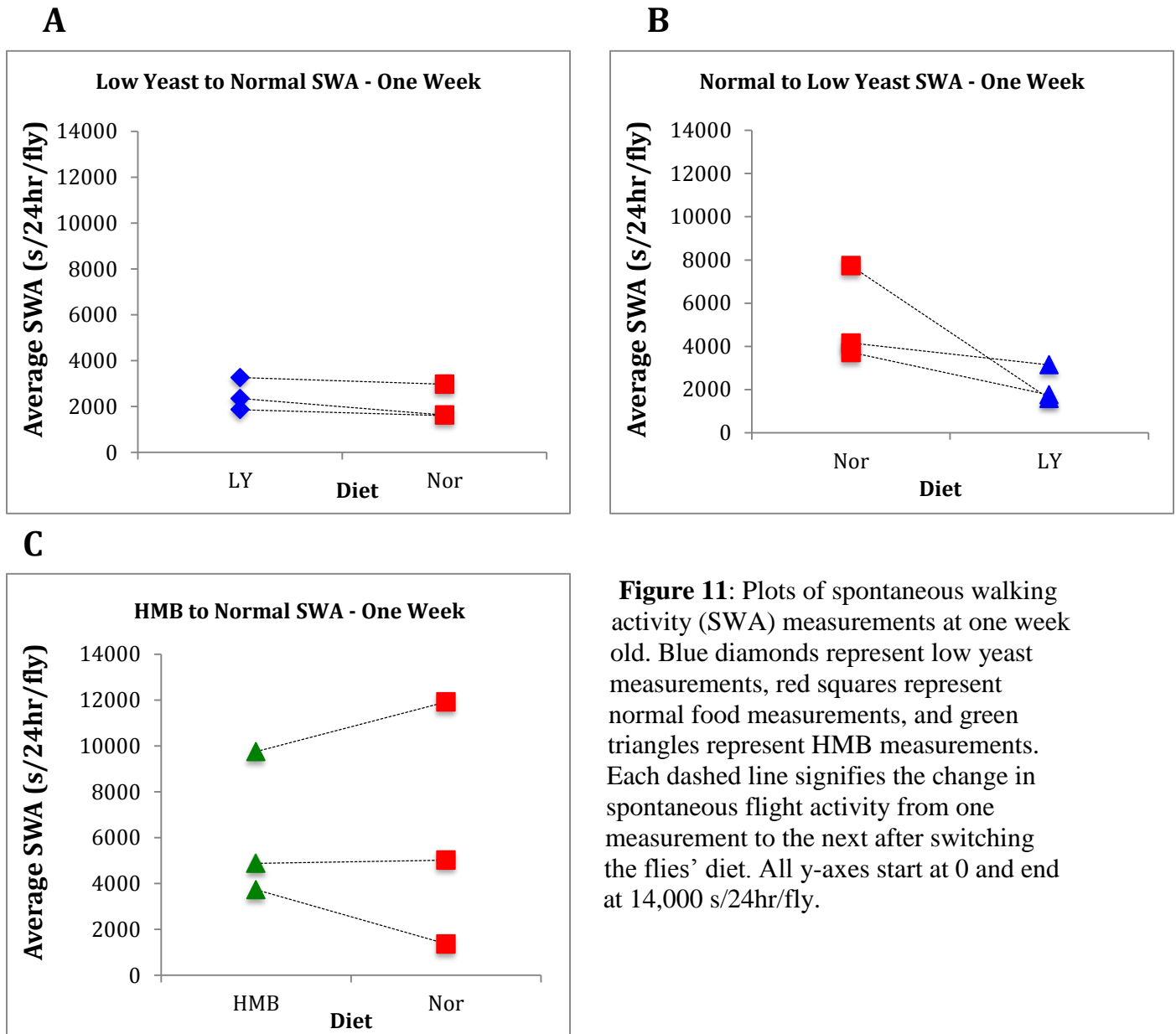


Figure 11: Plots of spontaneous walking activity (SWA) measurements at one week old. Blue diamonds represent low yeast measurements, red squares represent normal food measurements, and green triangles represent HMB measurements. Each dashed line signifies the change in spontaneous flight activity from one measurement to the next after switching the flies' diet. All y-axes start at 0 and end at 14,000 s/24hr/fly.

Table II: Average spontaneous walking activity (SWA) times (in seconds per fly per 24 hours) for switching experiments at one week old. P-values from a paired t-test are given for each experiment.

SWA - 1Wk	Switched to	
Native Diet	LY	Nor
LY	Average LY = 2493, Average Nor = 2068; p = 0.103	
Nor	Average Nor = 5202, Average LY = 2158; p = 0.194	
HMB	Average HMB = 6741, Average Nor = 6645; p = 0.973	

Switching Diet Experiments: Five Weeks

In a comparison of flight by low yeast flies on low yeast food and then switched to normal food, there was no significant change in flight activity (207 ± 32.6 vs. 149 ± 37.6 ; $p = 0.102$) or walking activity (3740 ± 1304 vs. 2787 ± 978 ; $p = 0.116$) (Figure 12A and 13A, Table III and IV). Normal-fed flies that were switched to low yeast food showed no change in walking activity (7358 ± 3114 vs. 7490 ± 3011 ; $p = 0.840$) or flight activity (317 ± 63.1 vs. 419 ± 38.1 ; $p = 0.384$) (Figure 12B and 13B, Table III and IV). When switching from HMB to normal food, there was no significant difference in spontaneous flight activity (138 ± 57.5 vs. 117 ± 44.2 ; $p = 0.331$) or walking activity (1911 ± 645 vs. 2104 ± 472 ; $p = 0.330$) (Figure 12C and 13C, Table III and IV).

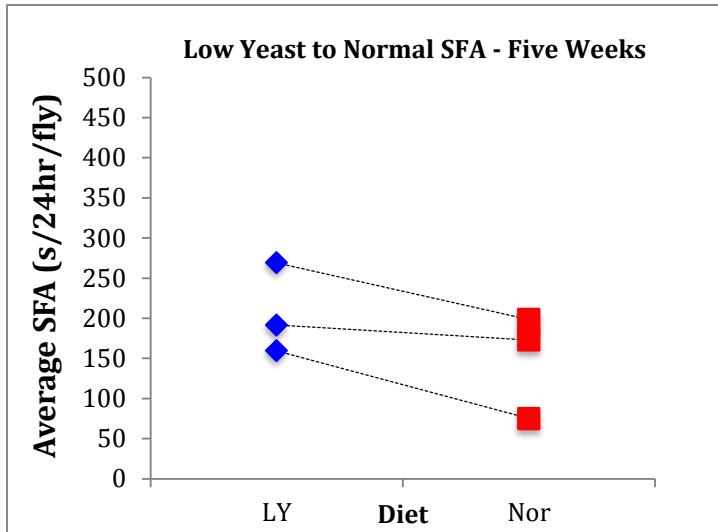
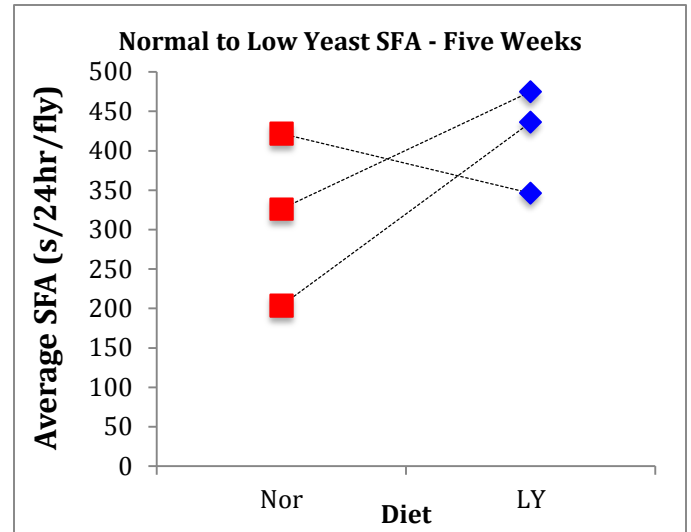
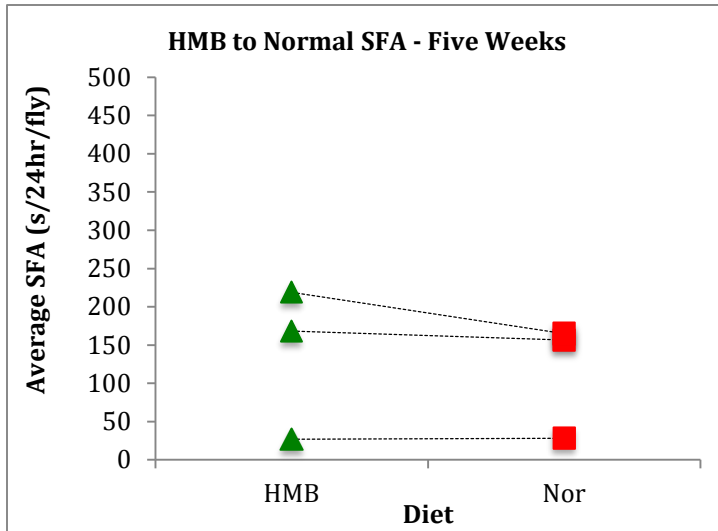
A**B****C**

Figure 12: Plots of spontaneous flying activity (SFA) measurements at five weeks old. Blue diamonds represent low yeast measurements, red squares represent normal food measurements, and green triangles represent HMB measurements. Each dashed line signifies the change in spontaneous flight activity from one measurement to the next after switching the flies' diet. All y-axes start at 0 and end at 500 s/24hr/fly.

Table III: Average spontaneous flying activity (SFA) times (in seconds per fly per 24 hours) for switching experiments at five weeks old. P-values from a paired t-test are given for each experiment.

SFA - 5Wks	Switched to	
Native Diet	LY	Nor
LY	NA	Average LY = 206.7, Average Nor = 148.9; p = 0.102
Nor	Average Nor = 317.3, Average LY = 419.0; p = 0.384	NA
HMB	NA	Average HMB = 137.9, Average Nor = 116.5; p = 0.331

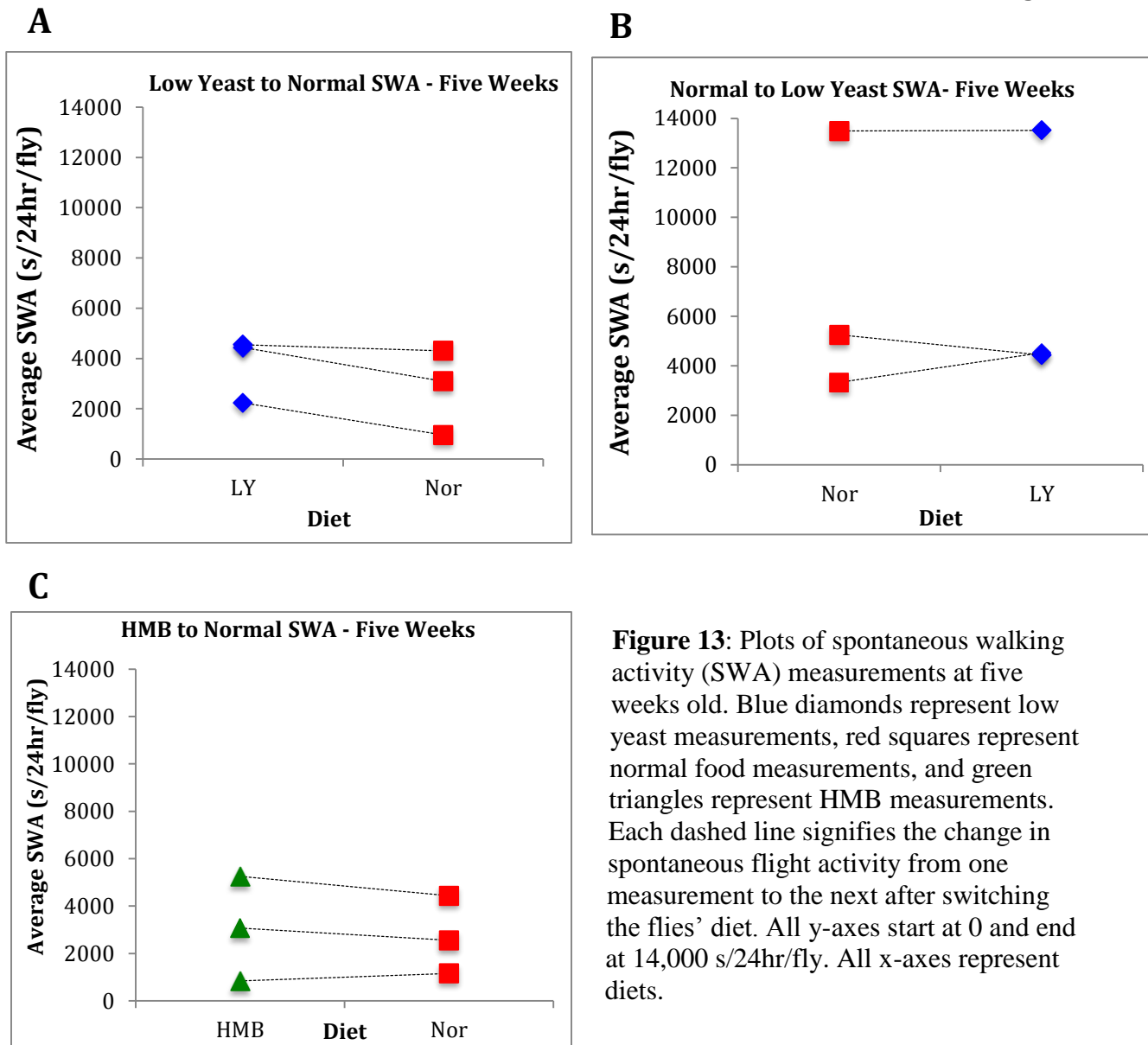


Figure 13: Plots of spontaneous walking activity (SWA) measurements at five weeks old. Blue diamonds represent low yeast measurements, red squares represent normal food measurements, and green triangles represent HMB measurements. Each dashed line signifies the change in spontaneous flight activity from one measurement to the next after switching the flies' diet. All y-axes start at 0 and end at 14,000 s/24hr/fly. All x-axes represent diets.

Table IV: Average spontaneous walking activity (SWA) times (in seconds per fly per 24 hours) for switching experiments at five weeks old. P-values from a paired t-test are given for each experiment.

SWA - 5Wks Native Diet	Switched to	
	LY	Nor
LY	NA	Average LY = 3740, Average Nor = 2787; p = 0.116
Nor	Average Nor = 7357, Average LY = 7490; p = 0.840	NA
HMB	NA	Average HMB = 1911, Average Nor = 2103; p = 0.330

Discussion

For this study, we had hypothesized that (i) HMB and dietary restriction will increase spontaneous flight and walking activity compared to a standard laboratory diet in young flies and that the effect of dietary restriction would be immediate; and (ii) HMB and dietary restriction will increase spontaneous flight and walking activity compared to a standard laboratory diet in old flies and that this effect would be more pronounced than that in younger flies. The results here do not support these hypotheses as there was no significant difference in spontaneous flight or walking activity between any of the dietary interventions, when switching the diet, or when comparing young and old flies.

The data collected in these experiments indicate that the increase in lifespan and health span with dietary restriction found in previous studies was not due to an increase in physical activity, either rigorous or moderate [26]. The data would also suggest that the increase in health span found with HMB supplementation in *Drosophila* is also not likely due to an increase in physical activity [5]. If the mechanism of action for these dietary interventions does include changes in physical activity, these are likely changes we were unable to measure with our apparatus. For example, very small changes in physical activity may not be significant enough to be statistically significant. Or, perhaps these changes in physical activity are not changes in walking and flying, but in wing or antennae movements, neither of which we are currently able to quantify.

We were surprised how little the flies flew and walked over the course of the 24-hour period. Flies typically only flew about 4 to 5 minutes and walked around 2 to 3 1/2 hours. This means each fly is stationary for least 20 hours of each day. This is likely due to the fact that the flies used in the lab have been in laboratory conditions for thousands

of generations (upwards of 75 years). These could be very different from flies found in the wild as these flies have not had any experience with a lack of food for many generations. It is possible that, if the effects of dietary restriction are due to increased movement in search of food, that lab-reared flies have lost this response. Also, since the activity of lab-raised flies has been restricted for so many generations, it is likely that they are not as healthy or as able to fly for long periods of time as wild-raised flies.

Conversely, small amounts of flight and walking may be normal for all flies. In the Ghimire et al paper, which measured total activity by counting the number of times flies broke any of a series of laser beams inside a vial, about 2,000 activities (beam breaks) / 10 flies / 24hrs were reported for normal fed flies [3]. This number increased to 6,000 activities / 10 flies / 24hrs when the concentration of yeast was decreased to 10% of normal (from 5% to 0.5% yeast). Firstly, this is a much larger difference than we were able to observe. This could be due to a larger decrease in yeast concentration than we had as our experiment only decreased yeast to 25%. However, our lab has previously found an increase in lifespan only decreasing yeast concentration to 25%, likely indicating that the mechanism of these effects either do not occur through an increase in physical activity or that the increase in physical activity was a type of an amount that our system was unable to measure.

Secondly, it is still not clear whether the activity being measured is walking, flying, or, possibly, flies moving back and forth in a beam. Our system would not be able to measure the latter, but it could, unlike the system used by Ghimire et al, distinguish between walking and flying activity. If we could find an accurate way to convert flight to

walking in terms of energy demand or oxidative damage we could add these measurements together to estimate total activity for each experimental group.

Dietary Restriction

1. Low yeast to normal diet

In switching one week-old low yeast flies to normal food, one of the low yeast measurements was quite a bit higher than the others, between three and four times as much time spent flying (Figure 10A). It is unclear why this measurement was so much higher than the others, but it could just have been by chance that the first two measurements were so close together. Even the highest low yeast measurement is not very large when compared to other flight measurements made from other treatments, some of which were 20 to 30 seconds higher this measurement. This trial had a significant decrease in flight time when switching to normal food, but the other two trials exhibited negligible change in flight time.

In trials switching five week-old low yeast flies to normal food, flight time consistently decreased (Figure 12 A). However, there was likely no significant difference between these groups due to the large variance between trials. For example, the third trial showed a decrease of about 80 seconds from low yeast to normal food. However, there was about a 100 second difference between the highest and lowest low yeast measurements and about a 120 second difference between the highest and lowest normal diet measurements. In the future, if variability between trials were minimized, a significant difference (specifically decreased flight activity) when changing diets may be observed.

When switching one week-old low yeast flies to normal food, spontaneous walking activity consistently decreased (Figure 11A). However, on average walking time only decreased 424 seconds. This may seem like a large decrease in walking time until variation is taken into consideration. There was a 1398 second difference between the highest and lowest walking measurements for low yeast food and a 1374 second difference between the highest and lowest walking measurements for normal food.

When switching five week-old low yeast flies to normal food, all three trials showed a decrease in spontaneous walking activity (Figure 13A). The average decrease in activity was 953 seconds. Once again, this decrease was much less than the variation amongst trial. There was a 2313 second difference between the highest and lowest walking measurements for low yeast food and a 3347 second difference between the highest and lowest walking measurements for normal food.

2. Normal to low yeast diet

When switching one week old normal-fed flies from normal to low yeast food, two of the trials exhibited an increased flight time by about 40 to 60 seconds, but one trial decreased by almost one hundred seconds (Figure 10B). This was likely due to the normal food being quite dry for this trial, affecting how easily the flies can eat the food. Flies likely were unable to eat the dry food as easily and therefore ate less. This could have made the amount of activity done by these flies much greater.

When switching five week-old normal-fed flies to low yeast food, two of the trials showed an increase in flight time, but one trial showed a decrease (Figure 12B). The trial finding a decrease in activity may have been a bit skewed as there was about an extra six

hours between the first 24 hours of measurement and the second 24 hours of measurement. This allowed for extra time for the flies to further equilibrate to the chamber, which may have lead to a decreased amount of flight.

One week-old normal diet flies that were switched to low yeast food also exhibited a decrease in walking time with every trial (average of 3044 seconds) (Figure 11B). However, this decrease was also relatively small when compared to variation between trials. There was a 4014 second difference between the highest and lowest walking measurements for normal food and a 1566 second difference between the highest and lowest walking measurements for low yeast food.

Five week-old normal diet flies switched to low yeast food exhibited minimal change in walking time (Figure 13B). This is similar to what happened when switching HMB flies to a normal diet. The very small changes for both of these switching experiments may indicate that, if either dietary restriction or HMB have any effect on spontaneous activity, these effects are less in older flies than in younger flies.

3. Conclusions

In general, spontaneous activity seemed to decrease from first measurements to second measurements. This could indicate that flies are still equilibrating to the chamber. In the future, more than 24 hours may be needed for equilibration to the chamber.

The lack of a significant difference in either spontaneous walking or flying activity between flies fed a low yeast (restricted) and normal (non-restricted) diet may indicate that the effects of dietary restriction do not involve changes in spontaneous activity. Considering our lab has previously shown that a 75% decrease in yeast

concentration was able to extend the lifespan and attenuate flight loss in *Drosophila*, these effects may not have occurred due to a change in spontaneous activity.

Since it is known that restricted diets increase sirtuin activity, it may be interesting to observe the spontaneous activity of flies with an overexpression of dSir2 or activation of dSir2 by the drug resveratrol. If these flies do exhibit a change in spontaneous activity, then changes in activity are likely part of the mechanism by which sirtuins have effects. The amount we decreased protein concentration in the food may have not been sufficient to increase sirtuin activity enough to cause an observable change in spontaneous activity. In the future, the amount of spontaneous activity with either a more drastic decrease in protein (10% or 5%) or a complete removal of food should be observed.

HMB

All trials switching one week-old flies fed an HMB-supplemented diet to a normal diet showed very little difference between initial and switched measurements, with differences only ranging from 0.2 to 18 seconds (Figure 10C). This appears to be strong evidence for no immediate difference in spontaneous walking or flight activity when HMB is removed from the diet. This could possibly be due to the fact that mTOR activates S6K1, which can cause upstream inhibition of Akt. Akt is then no longer able to activate mTOR. This negative feedback could have decreased the effects of HMB, even after just one week, and caused no change in physical activity when removing HMB. Conversely, if the loss of HMB-induced upregulation of mTOR did occur with the removal of HMB in the diet, then mTOR signaling had no observable effects on

spontaneous activity. It is also possible that 24 hours was not enough time for mTOR to be downregulated by the removal of HMB in the diet.

Five week-old HMB flies that were switched to a normal diet also showed very little change in flight activity, with differences in flight averaging 21 seconds (Figure 12C). This is consistent with the findings in one week-old flies, indicating that an immediate removal of HMB does not have an effect on spontaneous flight in young or old flies. Considering previous findings that HMB attenuated flight loss with age, these data suggest that this effect is not through changes in spontaneous flight time.

Neither one week-old nor five week-old HMB flies exhibited large changes in walking time when switched to normal food (Figure 11C, Figure 13C). This is consistent with the findings for flight time, indicating that, in addition to no effect on vigorous activity, HMB also has no effect on moderate activity.

Possible Sources of Error

The chamber for measurement of spontaneous activity was kept in the lab. Due to this fact, it was difficult to have much control over the temperature inside the chamber as the temperature of the lab room fluctuated quite significantly. The temperature in the chamber likely changed along with the temperatures in the lab, likely having an effect on the amount of activity done by flies as they do not fly as much in cold environments [45]. It is also possible that changes in temperature had an effect on the food the flies were fed, possibly making it more difficult to eat and accidentally creating reduced intake.

Also, by having the chamber in the lab, the amount of external sound and vibration could not be controlled. It is possible that loud noises and vibrations made flies fly more than they would on their own free will. In the future, if these studies were to be conducted in an incubator. The incubator should also be sound proof and kept away from human activities and movements.

Another problem arose when we looked at the fact that, quite consistently, flight and walking times seemed to decrease in the second 24-hr measurement, regardless of diet. This could possibly indicate that flies need more time to equilibrate in the chamber.

Future Studies

In order to decrease the variation in measurements between trials, it would be best to have as consistent environmental conditions as possible. In order to achieve this, it would be best to run experiments inside of an incubator with constant temperature and free from ambient sounds and vibrations. If this cannot be done, the spontaneous activity of two identical chambers of an equal number of flies raised in the same fashion could be measured side-by-side. Food would be switched in only one of the chambers while the other remained a control. By running these two chambers in tandem, any changes in activity simply due to temperature, ambient sound, or flies becoming accustomed to their new, larger living space, could be accounted for. This would help narrow down how much of any observed changes in activity were due to changes in diet.

For each treatment it would also be beneficial to run more than just three trials. Due to such a small number of trials in this study, any outliers had a much larger effect on the average flight or walking time for all three trials than they would have had with five or

ten trials. These ten trials could also be spread out across the year in case there are any seasonal changes in the flies' spontaneous activity.

With dietary restriction, since we were unable to observe a significant change in spontaneous activity with a 75% reduction in yeast, future studies could try food with no yeast or, better yet, a complete removal of food. This would help to see if the flies would be able to respond to more extreme dietary restriction.

To test if the flies we observed did not fly or walk much overall due to having been in laboratory conditions for several generations, some studies should be conducted on wild-caught *Drosophila melanogaster*, or possibly even other species of flies. Their responses to dietary restriction, HMB supplementation, and complete removal of food should be tested and compared to that of laboratory-raised flies.

If the increase in spontaneous activity previously observed with dietary restriction is due to flies moving more in search of food, then it is possible that flies only fly more during one specific part of the day (feeding time) and fly much less the rest of the day, possibly to conserve energy. This would yield a smaller total flight over the course of the day. Our method of quantifying spontaneous activity only found the average flight over the course of an entire day. However, from the MatLab data acquired, we can make a plot of amount of spontaneous activity versus time of day. These graphs would be interesting to produce and analyze, seeing if there is any significant change in spontaneous activity during specific times of the day.

To investigate if the actual upregulation of mTOR or sirtuin pathways has any effect on spontaneous activity in *Drosophila*, mutant lines with constitutively active or inactive mTOR or sirtuin pathways can be created. Spontaneous walking and flying can

be measured for these flies at one week and at five weeks after eclosion. If these mutants show no change in spontaneous activity compared to wild-type flies, then this may help explain why changes in these pathways with dietary interventions had no effect on spontaneous activity.

References

- [1] Mair, William, and Andrew Dillin. "Aging and survival: the genetics of life span extension by dietary restriction." *Annu. Rev. Biochem.* 77 (2008): 727-754.
- [2] Kaeberlein, Matt, Mitch McVey, and Leonard Guarente. "The SIR2/3/4 complex and SIR2 alone promote longevity in *Saccharomyces cerevisiae* by two different mechanisms." *Genes & development* 13.19 (1999): 2570-2580.
- [3] Ghimire, Saurav, and Man Su Kim. "Enhanced Locomotor Activity Is Required to Exert Dietary Restriction-Dependent Increase of Stress Resistance in *Drosophila*." *Oxidative medicine and cellular longevity* 2015 (2015).
- [4] Wilson, Jacob M., et al. " β -Hydroxy- β -methylbutyrate free acid reduces markers of exercise-induced muscle damage and improves recovery in resistance-trained men." *British Journal of Nutrition* 110.03 (2013): 538-544.
- [5] Beattie, Alexandra, et al. " β -Hydroxy- β -methylbutyrate extends lifespan and attenuates age-dependent loss of flight ability in *Drosophila melanogaster*." *The FASEB Journal* 28:1 (2014): 246.242.
- [6] Partridge, Linda, Matthew DW Piper, and William Mair. "Dietary restriction in *Drosophila*." *Mechanisms of ageing and development* 126.9 (2005): 938-950.
- [7] Kevei, Éva, and Thorsten Hoppe. "Ubiquitin sets the timer: impacts on aging and longevity." *Nature* 201 (2014): 4.
- [8] Adams, Julian. "The proteasome: structure, function, and role in the cell." *Cancer treatment reviews* 29 (2003): 3-9.

- [9] Eskelinen, Eeva-Liisa, and Paul Saftig. "Autophagy: a lysosomal degradation pathway with a central role in health and disease." *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research* 1793.4 (2009): 664-673.
- [10] Cornu, Marion, Verena Albert, and Michael N. Hall. "mTOR in aging, metabolism, and cancer." *Current opinion in genetics & development* 23.1 (2013): 53-62.
- [11] Partridge, Linda, et al. "Ageing in *Drosophila*: the role of the insulin/Igf and TOR signaling network." *Experimental gerontology* 46.5 (2011): 376-381.
- [12] Kapahi, Pankaj, et al. "Regulation of lifespan in *Drosophila* by modulation of genes in the TOR signaling pathway." *Current Biology* 14.10 (2004): 885-890.
- [13] Kaeberlein, Matt, et al. "Regulation of yeast replicative life span by TOR and Sch9 in response to nutrients." *Science* 310.5751 (2005): 1193-1196.
- [14] Powers, R. Wilson, et al. "Extension of chronological life span in yeast by decreased TOR pathway signaling." *Genes & development* 20.2 (2006): 174-184.
- [15] Schiaffino, Stefano, and Cristina Mammucari. "Regulation of skeletal muscle growth by the IGF1-Akt/PKB pathway: insights from genetic models." *Skeletal muscle* 1.1 (2011): 1.
- [16] Finkel, Toren, Chu-Xia Deng, and Raul Mostoslavsky. "Recent progress in the biology and physiology of sirtuins." *Nature* 460.7255 (2009): 587-591.
- [17] Oellerich, Mark, and Michael Potente. "FOXOs and sirtuins in vascular growth, maintenance, and aging." *Circulation Research* 110.9 (2012): 1238-1251.
- [18] Hong, Sungki, et al. "Cross-talk between sirtuin and mammalian target of rapamycin complex 1 (mTORC1) signaling in the regulation of S6 kinase 1 (S6K1) phosphorylation." *Journal of Biological Chemistry* 289.19 (2014): 13132-13141.

- [19] Tissenbaum, Heidi A., and Leonard Guarente. "Increased dosage of a sir-2 gene extends lifespan in *Caenorhabditis elegans*." *Nature* 410.6825 (2001): 227-230.
- [20] Viswanathan, Mohan, et al. "A role for SIR-2.1 regulation of ER stress response genes in determining *C. elegans* life span." *Developmental cell* 9.5 (2005): 605-615.
- [21] Rogina, Blanka, and Stephen L. Helfand. "Sir2 mediates longevity in the fly through a pathway related to calorie restriction." *Proceedings of the National Academy of Sciences of the United States of America* 101.45 (2004): 15998-16003.
- [22] Lee, In Hye, et al. "A role for the NAD-dependent deacetylase Sirt1 in the regulation of autophagy." *Proceedings of the National Academy of Sciences* 105.9 (2008): 3374-3379.
- [23] Jia, Kailiang, and Beth Levine. "Autophagy is required for dietary restriction-mediated life span extension in *C. elegans*." *Autophagy* 3.6 (2007): 597-599.
- [24] Tavernarakis, Nektarios, et al. "The effects of p53 on whole organism longevity are mediated by autophagy." *Autophagy* 4.7 (2008): 870-873.
- [25] Parashar, Vijay, and Blanka Rogina. "dSir2 mediates the increased spontaneous physical activity in flies on calorie restriction." *Aging* 1.6 (2009): 529.
- [26] Stanfel, Monique N., et al. "The TOR pathway comes of age." *Biochimica et Biophysica Acta (BBA)-General Subjects* 1790.10 (2009): 1067-1074.
- [27] Demontis, Fabio, and Norbert Perrimon. "FOXO/4E-BP signaling in *Drosophila* muscles regulates organism-wide proteostasis during aging." *Cell* 143.5 (2010): 813-825.
- [28] Howitz, Konrad T., et al. "Small molecule activators of sirtuins extend *Saccharomyces cerevisiae* lifespan." *Nature* 425.6954 (2003): 191-196.

- [29] Duffy, Peter H., et al. "Effect of chronic caloric restriction on physiological variables related to energy metabolism in the male Fischer 344 rat." *Mechanisms of ageing and development* 48.2 (1989): 117-133.
- [30] Weed, J. L., et al. "Activity measures in rhesus monkeys on long-term calorie restriction." *Physiology & behavior* 62.1 (1997): 97-103.
- [31] Deutz, Nicolaas E., et al. "Readmission and mortality in malnourished, older, hospitalized adults treated with a specialized oral nutritional supplement: A randomized clinical trial." *Clinical Nutrition* (2016).
- [32] Park, Bong-Sup, et al. "HMB attenuates muscle loss during sustained energy deficit induced by calorie restriction and endurance exercise." *Metabolism* 62.12 (2013): 1718-1729.
- [33] Eley, Helen L., Steven T. Russell, and Michael J. Tisdale. "Mechanism of attenuation of muscle protein degradation induced by tumor necrosis factor- α and angiotensin II by β -hydroxy- β -methylbutyrate." *American Journal of Physiology-Endocrinology and Metabolism* 295.6 (2008): E1417-E1426.
- [34] Roubenoff, R. "Sarcopenia and its implications for the elderly." *European journal of clinical nutrition* 54 (2000): S40-7.
- [35] Blagosklonny, Mikhail V. "Calorie restriction: decelerating mTOR-driven aging from cells to organisms (including humans)." *Cell Cycle* 9.4 (2010): 683-688.
- [36] Mair, W., et al. "Calories do not explain extension of life span by dietary restriction in *Drosophila*." *PLoS Biol* 3.7 (2005): e223.
- [37] Martin, C. K., et al. "Effect of calorie restriction on resting metabolic rate and spontaneous physical activity." *Obesity* 15.12 (2007): 2964-2973.

- [38] Garland, T., et al. "The biological control of voluntary exercise, spontaneous physical activity and daily energy expenditure in relation to obesity: human and rodent perspectives." *The Journal of experimental biology* 214:2 (2011): 206-229.
- [39] Magwere, Tapiwanashe, et al. "Flight activity, mortality rates, and lipoxidative damage in *Drosophila*." *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences* 61.2 (2006): 136-145.
- [40] Roberts, D. B. "*Drosophila melanogaster*: the model organism." *Entomologia experimentalis et applicata* 121.2 (2006): 93-103.
- [41] Owusu-Ansah, E. and N. Perrimon. "Modeling metabolic homeostasis and nutrient sensing in *Drosophila*: implications for aging and metabolic diseases." *Disease Models and Mechanisms* 7.3 (2014): 343-350.
- [42] Coquin, L., et al. "Metabolomic and flux - balance analysis of age - related decline of hypoxia tolerance in *Drosophila* muscle tissue." *Molecular systems biology* 4.1 (2008): 233.
- [43] Sarbassov, Dos D., et al. "Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex." *Science* 307.5712 (2005): 1098-1101.
- [44] Weinert, Brian T., et al. "Proteome-wide mapping of the *Drosophila* acetylome demonstrates a high degree of conservation of lysine acetylation." *Sci. Signal.* 4.183 (2011): ra48-ra48.
- [45] Dillon, Michael E., and Melanie R. Frazier. "*Drosophila melanogaster* locomotion in cold thin air." *Journal of Experimental Biology* 209.2 (2006): 364-371.

Drosophila Mitochondrial Isolation and Seahorse Mito Stress Assay Procedure

Previous Evening:

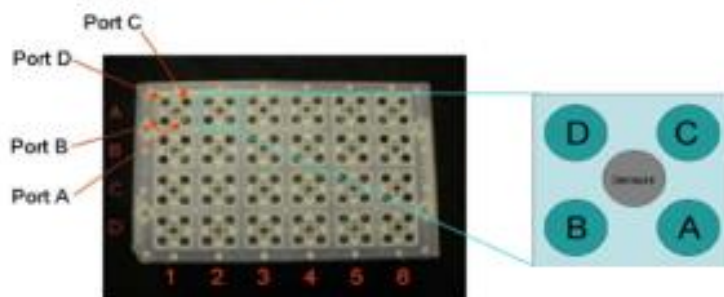
- 1) Prepare Isolation and Respiration Buffers
- 2) Prepare reagents (ADP, Oligomycin, FCCP, Antimycin A or Rotenone) in 2x Respiration Buffer.

Morning of Experiment: Heat Respiration Buffer to 37 °C, add 10 mM succinate, and pH to 7.2 with KOH.

All procedures performed at 4 °C

- 1) Dissect 50 flies per treatment over ice - separate head, wings, and abdomen from thoraces.
- 2) Place tissue in a 2 mL epi tube with 1 mL isolation buffer
- 3) Transfer to glass homogenizer vessel (cut tip of 1 mL pipette tips to keep from clogging). Add additional 5 mL Isolation buffer.
- 4) Mince tissue briefly (2-3s) in Polytron and transfer solution to glass homogenizer vessel for Potter S Homogenizer.
- 5) Homogenize 10 min at slowest RPM and with gentle up and down motion.
- 6) Transfer homogenate evenly into 4 epi tubes.
- 7) Spin 10 min at 400 g (1600 RPM on Eppendorf 5415) in cold room.
- 8) Collect supernatant into 4 new epi tubes.
- 9) Discard myosin pellets.
- 10) Spin collected supernatant at 10,000g (1069 RPM on Eppendorf 5415) for 10 minutes and discard supernatant.
- 11) Resuspend pellets in 1 mL Isolation Buffer (combine all into 1 tube).
- 12) Spin at 10,000g (1069 RPM on Eppendorf 5415) for 10 minutes. Discard supernatant.
- 13) Resuspend final pellets in volume (in μ L) Respiration Buffer equal to 4x (μ L:number of flies).
- 14) Perform Bradford Assay to quantify protein yield in mito suspension.
- 15) Based on protein concentration, load 8 μ g/well in Seahorse XF 24 microplate, except wells A1, B4, C3, and D6, which will be used for background (see picture below).

microplate, except wells A1, B4, C3, and D6 which will be left for background (picture below).



XF24 Cartridge - Top View with injection ports layout

- 16) Add sufficient volume Respiration Buffer + 10mM succinate to equal 30 μL /well.
- 17) Centrifuge using swinging bucket centrifuge at 4°C, 2000g for 20 min (3408 RPM on Eppendorf 5415). Make sure to balance properly.
- 18) Fill cartridge with A: 50 μL ADP (40mM), B: 55 μL Oligomycin (32 μM), C: 60 μL FCCP (40 μM), D: 65 μL Antimycin A (40 μM) or Rotenone (20 μM) per oxidative stress protocol.
- 19) Following centrifugation, fill wells with 470 μL Respiration Buffer to reach final volume of 500 μL .
- 20) Begin calibration procedure on Seahorse using utility plate. Ensure utility plate and cartridge are loaded properly, with notched corner aligned with the front right corner of the XF 24 tray (see photo below).



- 21) Allow culture plate to approach room temperature during calibration (about 10 min).
- 22) Assign wells and run Mito Stress test protocol.
- 23) Replace utility plate with culture plate and begin Mito Stress Assay.

Development of Seahorse Mito Stress Assay for *Drosophila*

First Attempt – November 17, 2015

For the first attempt, we decided to follow the same procedure as had previously been used for mammalian tissue [1,2]. We compared normal flies to those fed paraquat, hoping to see reduced mitochondrial function (decreased basal and maximal oxygen consumption) in the paraquat group. The result was a high rate of oxygen consumption for both groups, with no response to the addition of ADP. We hypothesized that the ionic strength of the isolation buffer being used was too strong. Previous experiments extracting mitochondria from *Drosophila* had used buffers mainly comprised of sugar (mannitol and sucrose) with very little salt. It is possible that the higher ionic strength of the isolation buffer had caused the mitochondria to lyse and the respiration we were measuring was from free cytochrome c molecules.

Second Attempt – February 11, 2016

We prepared an isolation buffer (below) using the protocol from Tong and Williams 2007 [3].

- Isolation Buffer: 225 mM mannitol, 75 mM sucrose, 10 mM MOPS, 1 mM EGTA, 0.5% BSA, pH 7.2 at 4°C 38.

For this attempt, we compared dissected thoraces to non-dissected flies. For both groups, little to no oxygen consumption was detected. We had predicted this was because we did not have enough mitochondria in our sample as we had to scrape some of the muscle tissue from one of the pellets. It was also possible that the protease (subtilisin) had broken damaged the mitochondria.

Third Attempt – March 4, 2016

We prepared both isolation (from second attempt) and respiration (below) buffers using the protocol from Tong and Williams 2007 [3].

- Respiration Buffer: 225 mM mannitol, 75 mM sucrose, 10 mM KCl, 10 mM Tris-HCl, 5 mM KH₂PO₄, pH 7.2 at 25°C.

The first treatment was thoraces without protease and separated from chitin with a slower centrifugation (400g instead of 720g, which had created a bilayer pellet with a black layer at the bottom, likely of chitin, and a white layer on top, likely of muscle tissue). This slower speed left more muscle tissue in the

supernatant, proving to be effective. The second treatment was non-dissected flies with protease and separated with paper filtration. The filtration ended up losing too much of the sample, proving ineffective.

Both groups ended up having a relatively low basal oxygen consumption rate, which exhibited very small changes with the addition of ADP. We realized this was likely due to the absence of substrate in the respiration buffer. The previous respiration buffer had succinate and a very small amount of rotenone.

Fourth Attempt – March 29, 2016

For this attempt, we used the same isolation and respiration buffers as before, but added 10 mM succinate and 2 μ M rotenone to one treatment and no substrate to the other treatment. This was to test if the addition of substrate would have any effect on respiration. What we found was that the treatment with no substrate had the same result as before, having a relatively low basal oxygen consumption rate and very small changes with the addition of ADP, but the treatment with succinate and rotenone had little to no basal oxygen consumption and no changes with the addition of ADP.

We predicted that the rotenone was likely causing this lack of basal oxygen consumption. Rotenone acts by inhibiting the transfer of electrons from complex I to ubiquinone and is used in mammalian assays to increase the proton gradient due to fewer protons entering the mitochondrial matrix through complex I. This increased proton gradient increases the proton flow through complex III. Complex II, or succinate dehydrogenase, oxidizes succinate and transfers the electrons to ubiquinone, which then transfers electrons to complex III. Therefore, the addition of rotenone actually increases the rate of electron transfer from succinate to complex III.

However, it is possible that, due to differences in protein structures of the electron transport chain between insects and mammals, that rotenone instead caused an inhibition of respiration, even at such a low concentration. This difference may be part of the reason why rotenone is a common insecticide.

Fifth Attempt – April 11, 2016

For this attempt, we compared using 10 mM succinate to using 10 mM pyruvate and 2 mM malate. This was to see if just succinate alone, without rotenone, would be an effective substrate for the assay, or if pyruvate and malate would be more effective. We found odd oxygen consumption rates and oxygen concentration values (some were even negative, which is impossible). This was likely due to problems with the Seahorse XF 24 as the machine had made a strange, loud noise when first turning it on. For the next attempt, the same procedure should be used, but making sure any problems with the Seahorse XF 24 have been resolved.

References

- [1] Lanza, Ian R., and K. Sreekumaran Nair. "Muscle mitochondrial changes with aging and exercise." *The American journal of clinical nutrition* 89.1 (2009): 467S-471S.
- [2] Zid, Brian M., et al. "4E-BP extends lifespan upon dietary restriction by enhancing mitochondrial activity in *Drosophila*." *Cell* 139.1 (2009): 149-160.
- [3] Tong, James Jiayuan. "Mitochondrial delivery is essential for synaptic potentiation." *The Biological Bulletin* 212.2 (2007): 169-175.